Bioorganic & Medicinal Chemistry 21 (2013) 5012–5020

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Exploring pyrimidine-substituted curcumin analogues: Design, synthesis and effects on EGFR signaling

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ARTICLE INFO

Article history:
Received 9 May 2013
Revised 21 June 2013
Accepted 22 June 2013
Available online 2 July 2013

Keywords:
Apoptosis
Cell cycle
Colon cancer
Curcumin analogues
EGFR

ABSTRACT

Epidermal growth factor receptor (EGFR) is an effective molecular target of anti-cancer therapies. Curcumin inhibits cancer cell growth in vitro by suppressing gene expression of EGFR and reduces tumor growth in various animal models. To overcome instable and insoluble properties of curcumin as therapeutics, we designed and synthesized six novel pyrimidine-substituted curcumin analogues with or without a hydroxyl group originally present in curcumin. The cell viability tests indicated that IC_{50} of the analogues containing hydroxyl group were 3 to 8-fold lower than those of the analogues without hydroxyl group in two colon cancer cell lines tested. Western blot analysis indicates the analogues containing hydroxyl group inhibited expression and tyrosine phosphorylation of EGFR. Further protein analyses showed that the analogues had anti-cellular proliferation, pro-apoptosis, and cell cycle arrest properties associated with suppressed EGFR expression. These results indicate that the hydroxyl groups in curcumin and the analogues were critical for observed biological activities.

1. Introduction

Epithelial growth factor receptor family with its most prominent member of EGFR represents valid targets for anti-cancer therapy. Anti-EGFRs MoAbs (cetuximab, panitumumab, and trastuzumab) and TKIs (gefitinib, erlotinib, and lapatinib) have now been approved for the treatment of advanced colorectal cancer, squamous cell carcinoma of the head and neck, advanced non small cell lung cancer, as well as pancreatic and breast cancer because majority of human cancers are originated from accumulated genetic mutations of epithelia cells and many types of human cancers are associated with over-expressed EGFR.[1] However, the benefits of costly TKIs and anti-EGFRs MoAbs treatment to most cancer patients are very limited due to complications of drug-resistant and side effects. The median survival rates are only a few months more than those patients treated with conventional therapies. Hence there is a great need in developing inexpensive and more effective anti-EGFR drugs.

Curcumin, a compound that is responsible for tumeric therapeutic properties in traditional medicine, has been shown to inhibit human colon cancer cell growth by suppressing gene expression of EGFR through reducing the trans-activation activity of Egr-1.[2] Curcumin also inhibits a variety of tumor growth in various animal models such as mammary adenocarcinoma, stomach, duodenal in addition to colon cancers.[3–6] Therefore, understanding the relationship between structure and biological activities of curcumin will be important for developing novel anti-EGFR drugs.

Curcumin (Fig. 1) consists of a phenolic ring, alternating double bond and β-dicarbonyl group, which form a conjugated π bond skeleton,[7] wherein the β-dicarbonyl group and substituted phenol rings are the pharmacophore.[8–12] Curcumin can exist in several tautomeric forms, including a 1,3-diketo form and 2 equiv enolate forms. The enolate form is more energetically stable in the solid phase and in solution.[13] Curcumin has poor water solubility due to its rigid and planarity structure, which affects its absorption from digestive system into blood circulation.[14] Moreover, the β-dicarbonyl group in curcumin is unstable and easily decomposed, which leads to an even lower blood concentration and further reduces pharmacological potency of curcumin.[14] Indeed, turmeric containing ~5% curcumin is pharmacological safe even when consumed up to 100 g per day in diet.[15,16] Thus, we have designed and synthesized six novel pyrimidine-substituted curcumin analogues with or without hydroxyl group in which the plane conjugate structure of curcumin was retained while the chemical

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0968-0896
http://dx.doi.org/10.1016/j.bmc.2013.06.053

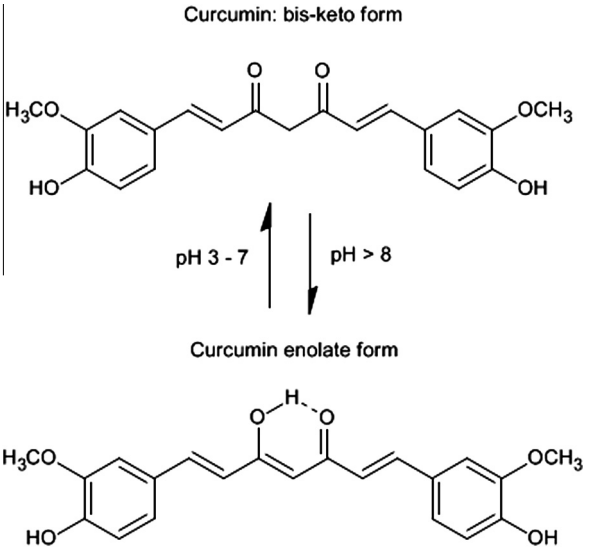


Figure 1. Chemical structure of curcumin.

stability and solubility of the analogues were greatly improved (Fig. 2).[17]

Colorectal cancer is one of the leading causes of cancer death in the developed Western countries[18] and increases in an alarming rate in Asian.[19] EGFR has been reported to be over-expressed from 25% to 82% in colorectal cancers.[20] Therefore, we used two colon cancer cell lines as a model system to understand the relationships between structure and biological activity of curcumin analogues towards EGFR regulations.

2. Results and discussion

2.1. Chemical synthesis of six novel primidine substituted curcumin analogues

To retain the plane conjugate structure and to improve the chemical stability and solubility in water and organic solvent of curcumin, we used the following scheme to synthesize the curcumin analogues (Scheme 1).

Reagents and conditions: (i) 4,6-dimethyl-2-hydroxyl-pyrimidine hydrochloride, HCl, EtOH, PhMe, 110 °C, 36 h, 65–70%. (ii) $POCl_3$, DIPEA, 110 °C, 12 h, 40–45%. (iii) Appropriate amine, EtOH, 90 °C, 12 h, 52–69%.

4,6-Dimethyl-2-hydroxyl-pyrimidine hydrochloride was synthesized in one-pot as previously reported.[17]

Compounds **4a–f** were synthesized following the convergent synthetic approach depicted in Scheme 1. The compounds **2a–b** were prepared from the appropriate aromatic aldehydes with 4,6-dimethyl-2-hydroxyl-pyrimidine hydrochloride according to the procedure we reported previously.[21]

Subsequently, the compounds **3a–b** were synthesized by refluxing a solution of **2a–b** with *N*,*N*-diethylaniline in an excess of $POCl_3$ overnight in the yields of 40–45%. In the last step, target compounds **4a–f** could be prepared by refluxing an ethanol solution of the appropriate amine with compounds **3a–b** in good yields (52–69%). Comparing with curcumin, the target compounds **4a–f** can be dissolved in polar solvent such as ethanol and in water with acid as well.

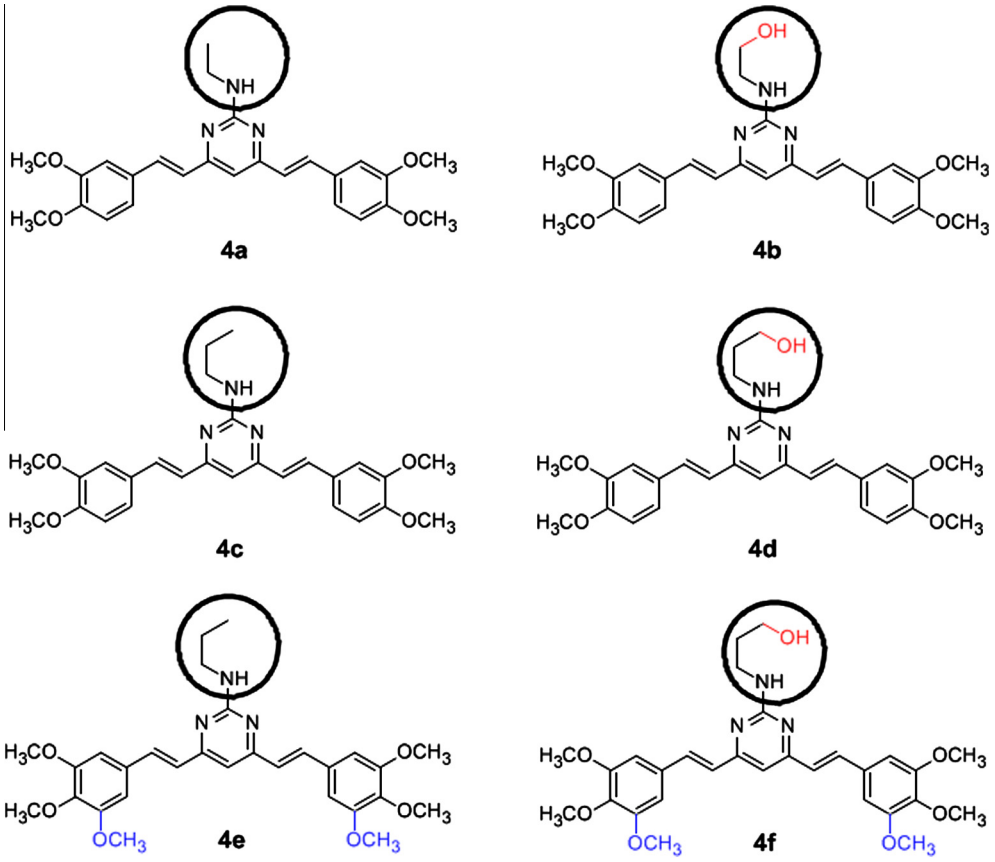
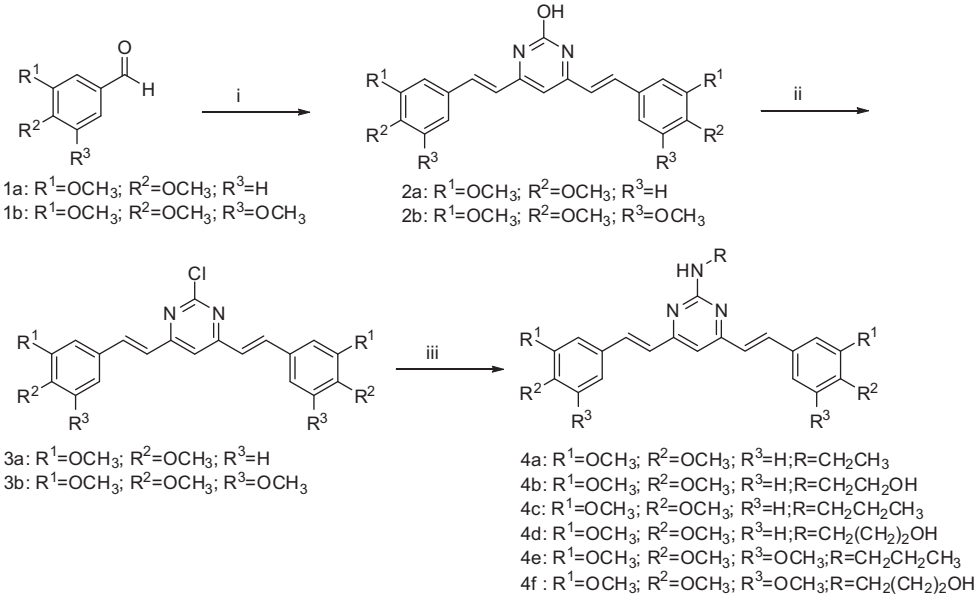


Figure 2. Chemical structures of **4a–f**.



Scheme 1. Synthesis of compounds **4a**–**f**.

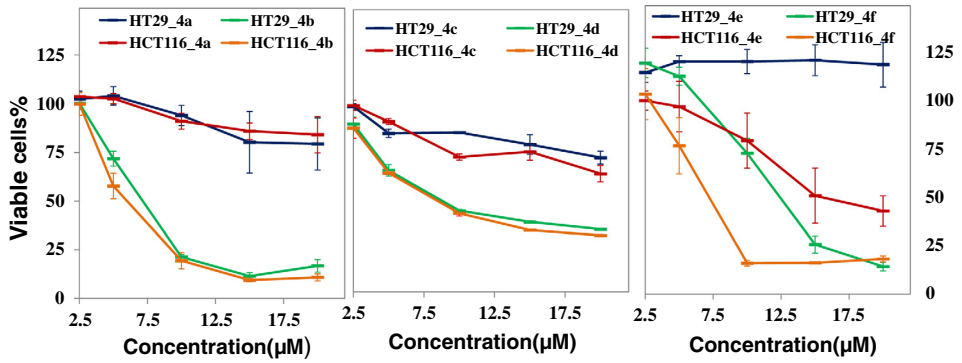


Figure 3. Growth inhibitory effect of **4a**–**f** on HCT116 and HT29 human colon cancer cells. HCT116 and HT29 were seeded in 96-well plates, respectively. After 24 h, cells were treated with serial concentrations of curcumin analogues. After 48 h of treatment, cell viability was measured by resazurin assay as described under Materials and Methods. Data are expressed as the mean ± standard deviation.

2.2. Curcumin analogues with hydroxyl group were more effective than their counterparts in inhibiting colon cancer cell growth

We compared the inhibitory effects of the six analogues on two colon cancer cell lines using resazurin assay. Three curcumin analogues with hydroxyl group were **4b**, **4d**, and **4f** and their corresponding curcumin analogues without hydroxyl group were **4a**, **4c**, and **4e**, respectively. In both HCT116 and HT29 cells, all five curcumin analogues except **4e** in HT29 showed dose-dependent inhibition on the cell growth and the analogues with hydroxyl group showed much stronger grow inhibition effects than their corresponding curcumin analogues without hydroxyl group (Fig. 3). For example, three curcumin analogues with hydroxyl group (**4b**, **4d**, and **4f**) showed IC_{50} from 6.2 to 12.8 μM in both cancer cells, while IC_{50} of the three analogues without hydroxyl group (**4a**, **4c**, and **4e**) were 3 to 8-fold higher (from 21.9 to >100 μM) (Table 1).

Table 1

Compound	IC_{50} ± SD[a] (μM)	
	HT29	HCT116
4a	38.5 ± 25.4	46.4 ± 23.6
4b	7.1 ± 0.4	6.2 ± 1.2
4c	>100	28.0 ± 12.7
4d	12.8 ± 1.9	9.9 ± 6.9
4e	>100	21.9 ± 7.0
4f	12.4 ± 0.9	7.4 ± 1.8

[a] IC_{50}, compound concentration required to inhibit cell proliferation by 50%.

2.3. Analogue 4b suppressed EGFR expression and phosphorylation

Curcumin is a component of turmeric. Turmeric is a spice derived from a member of ginger family. Traditional medicine uses turmeric for biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism, and sinusitis.[22] Modern research indicates curcumin is responsible for many of tumeric therapeutic properties. It is subsequently demonstrated that curcumin inhibits human colon cancer cell growth by suppressing gene expression of EGFR.[2]

Data in Figure 3 and Table 1 showed that the curcumin analogues inhibited the growth of two human colon cells. Since EGFR might be the most important growth signaling pathway in the two colon cancer cell lines, we then tested if the observed cytotoxicity

was due to suppressed EGFR expression and phosphorylation by the analogues.

As shown in Figure 4, after incubation with analogue **4a** or **4b** for 72 h in both colon cancer cells, **4a** at 20 μM concentration did not significantly decrease the levels of P-EGFR(Tyr1068) or EGFR in both of HCT116 and HT29 cells. In contrast, **4b** significantly decreased the levels of both phosphorylated P-EGFR(Tyr1068) and EGFR in a concentration-dependent manner.

2.4. Analogue 4b induced cell cycle arrest in a concentration-dependent manner

It was reported that curcumin inhibits HT29 colon cancer cell growth by suppressing gene expression of EGFR.[2] We found that the curcumin analogues inhibited EGFR expression and phosphorylation in both HT29 and HCT116 cells (Fig. 4). It was reported in literature that HCT116 cells treated with curcumin are largely accumulated in G2/M phase.[23] We thus tested how the curcumin analogues affect cell cycles in both HCT116 and HT29 cells. As shown in Figure 5A (HCT116 cells), **4a** at the concentration of 20 μM and **4b** at the concentrations of 5, 10, and 20 μM increased G2/M cell population to 1.03-, 1.54-, 1.78-, and 2.05-fold of that of the control cells, respectively. In HT29 cells (Fig. 5B), **4a** at the concentration of 20 μM and **4b** at the concentrations of 5, 10, and 20 μM increased G2/M cell population to 1.10-, 1.86-, 1.94-, and 2.40-fold of that of the control cells, respectively. Interestingly, **4b** at 5 μM concentration induced G0/G1 cell cycle arrest in both HCT116 and HT29 cells.

It was reported that curcumin induced G2/M cell cycle arrest is accompanied by increased $p21^{WAF-1/CIP-1}$ expression.[5] $p21^{WAF-1/CIP-1}$ is a potent cyclin-dependent kinase inhibitor (CKI). $p21^{WAF-1/CIP-1}$ binds to and inhibits the activity of cyclin-CDK2 or cyclin-CDK1 complexes, and thus functions as a regulator of cell cycle progression. Since **4b** at 5 μM concentration induced both G0/G1 and G2/M cell cycle arrests (Fig. 5A and B), we assessed cell cycle signaling checkpoint proteins, including $p21^{WAF-1/CIP-1}$, CDK2, CDK4, Cyclin D1, Cyclin E1, and Rb systematically in HCT116 cells after treating the cells with or without the analogues. Rb stands for retinoblastoma protein, which is a tumor suppressor protein that is dysfunctional in several major cancers. One function of Rb is to prevent excessive cell growth by inhibiting cell cycle progression.

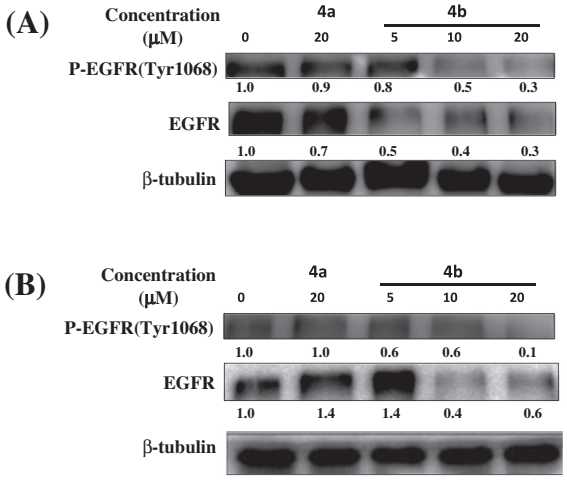


Figure 4. Effects of the curcumin analogues on EGFR and P-EGFR (Tyr1068) in human colon cancer cells HCT116 and HT29. Both cells were seeded in 15 cm dishes for 24 h, and then cells were treated with **4a** at 20 μM or **4b** at 5, 10 and 20 μM, respectively. After 72 h of incubation, cells were harvested for Western blotting analysis. The numbers underneath the blots represent band intensities (normalized to the loading controls, means of three independent experiments) measured by the Image J software. The standard deviations were all within ±15% of the means (data not shown). β-Tubulin was used as equal loading controls. The experiments were repeated for three times.

As shown in Figure 5C, **4a** at 20 μM and **4b** at 5, 10, and 20 μM concentrations induced significant $p21^{WAF-1/CIP-1}$ expression (20.0, 21.4, 28.6, and 25.7 fold-increases, respectively). Analogue **4a** at 20 μM did not induce significant changes in all other proteins tested. In contrast, **4b** inhibited expression of Cyclin D1, Cyclin E1, and Rb in a concentration-dependent manner even though **4b** did not induce significant changes in CDK2 and CDK4 proteins at all concentrations tested.

We concluded from the data in Figure 5 that the curcumin analogues, especially **4b**, caused cell cycle arrest in both HCT116 and HT29 human colon cancer cells by inducing significant $p21^{WAF-1/CIP-1}$ expression. Analogue **4b** also inhibited CyclinD1, CyclinE1, and Rb expression in a concentration-dependent manner.

2.5. Effects of the curcumin analogues on proteins involved in apoptosis

Studies indicated that intracellular caspases are one of the major signaling pathways involved in apoptotic cell death. Caspases are a family of structurally related cysteine proteases. For example, caspases-2, -3, -6, -7 and -9 can cleave poly (ADP ribose) polymerase (PARP).[24] Curcumin was reported to activate caspases 3, 8, and 9 in the colon cancer cell lines SW480 and SW620.[25]

We first examined the expression levels of activated caspase 3 and cleaved PARP because PARP is a substrate of caspase 3 after treating the HCT116 colon cancer cells with or without the analogues. As shown in Figure 6A, **4a** at 20 μM concentration did not cause any noticeable change in both caspase 3 and PARP. In contrast, **4b** caused activated caspase 3 and cleaved PARP in a concentration-dependent manner.

Heat shock proteins (HSP) are a class of functionally related proteins involved in the folding and unfolding of other proteins. Their expression is increased when cells are exposed to elevated temperatures or other stress including harmful chemicals. It has been established that HSP70 directly inhibits apoptosis whereas HSP90 stabilizes various growth factor receptors including EGFR. Hence inhibition of HSP90 may induce apoptosis.[26] We found that **4a** at 20 μM concentration had no effect on the expression of HSP-70 and HSP-90, while **4b** decreased the levels of HSP90 and HSP70 expressions in a concentration-dependent manner.

Bcl-2 is a family of evolutionarily related proteins. These proteins govern mitochondrial outer membrane permeabilization and can be either pro-apoptotic, such as Bax, or anti-apoptotic such as Bcl-2.[27] We measured the levels of Bcl-2 and Bax expression in HCT116 colon cancer cells after treating the cells with different concentrations of **4a** and **4b** for 72 h. Compound **4a** decreased slightly the level of anti-apoptotic Bcl-2 expression but increased the level of pro-apoptotic protein Bax expression (Fig. 6B). Compound **4b** had a stronger effect on the expression of Bcl-2 and Bax and the effect was concentration-dependent.

A quantification of BAX and Bcl-2 expression indicated that **4a** at 20 μM concentration increased the ratio of Bax/Bcl-2 by 1.57-fold compared to the untreated control, whereas **4b** increased the ratio of Bax/Bcl-2 by 2.1-, 17-, and 19-fold at 5, 10, and 20 μM concentrations, respectively. Therefore, the curcumin analogues worked on multiple protein targets to promote apoptosis in the human colon cell line HTC116, which was consistent with the multiple functions of EGFR signaling.

3. Conclusions

In summary, we describe the synthesis of six novel pyrimidine-substituted curcumin analogues with or without hydroxyl group in which the critical plane conjugate structure of curcumin was

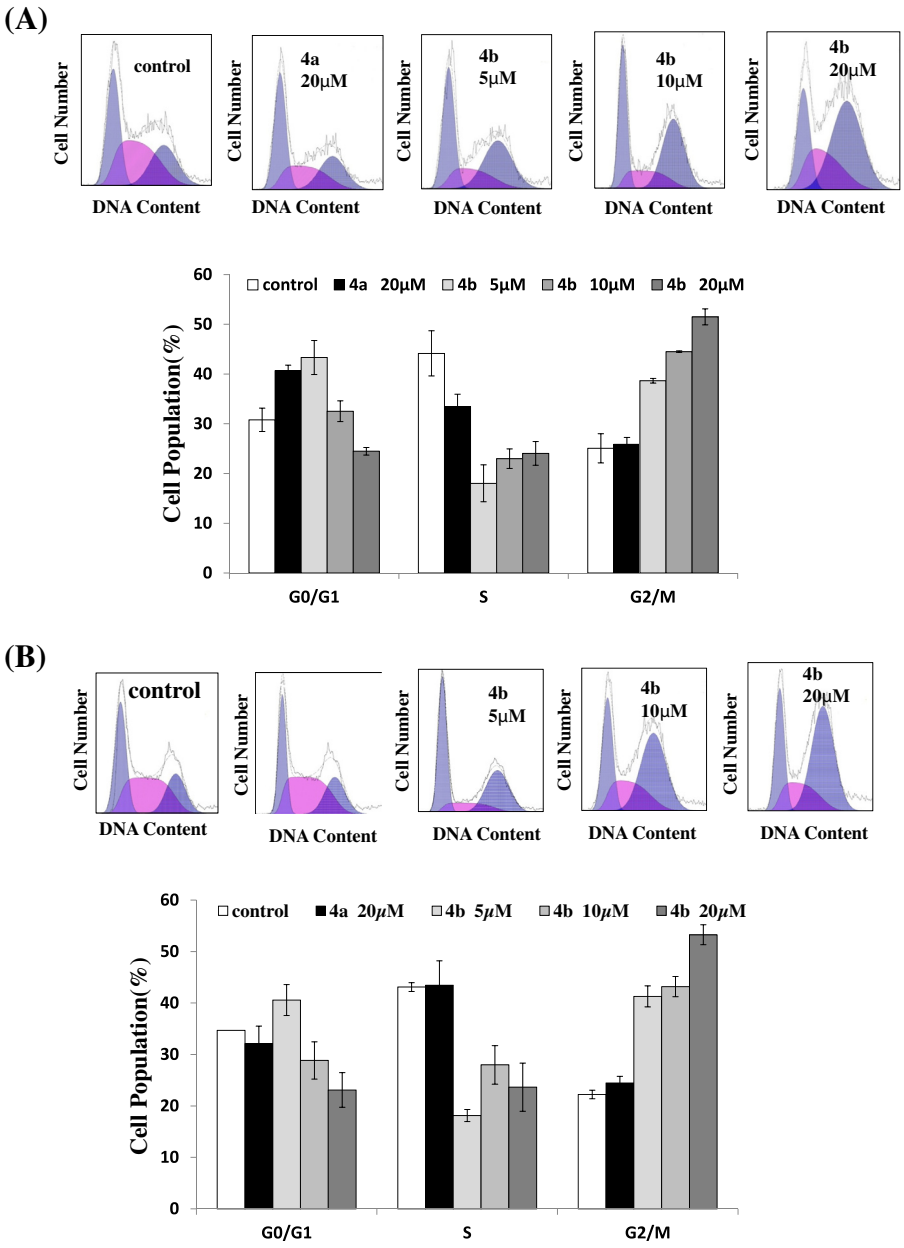


Figure 5. Cell cycle-related analysis of human colon cancer cells after treating with the curcumin analogues. HCT-116(A) and HT29 (B) cells after treating with the curcumin analogues **4a** or **4b**. The HCT116 and HT29 cells were seeded in 6-well plates (4×10^4/ml, 2 ml/well) for 24 h, and then the cells were treated with **4a** at 20 μM or **4b** at 5, 10 and 20 μM concentrations. After 24 h, cells were harvested and subject to cell cycle analyses as described in Section 4. All results shown are mean ± SD, and are representative of three independent assays. Statistical analyses were conducted among control and treated groups in G0/G1, S, and G2/M phases. (C) Effects of **4a** and **4b** on cell cycle related proteins in HCT116 human colon cancer cells.

retained while the chemical stability and solubility were improved. The cell viability tests indicated that IC_{50} of the three curcumin analogues containing hydroxyl group was 3 to 8-fold lower than that of the three analogues without hydroxyl group in two colon cancer cell lines tested. Western blot analysis indicates the analogues containing hydroxyl group inhibited expression and tyrosine phosphorylation of EGFR. Cell cycle and further cell cycle and apoptotic check point protein analyses showed that the analogues had expected anti-cellular proliferation, pro-apoptosis, and cell cycle arresting properties associated with suppressed EGFR expression and activation. These results suggest that the hydroxyl group in curcumin and the analogues (Fig. 7) might be critical for EGFR regulating activities.

4. Experimental section

4.1. Cell culture

Human colon cancer cells HCT116 and HT29 were obtained from Shanghai Cell Bank of Chinese Academy of Science (China) and were maintained in McCoy's 5A (Sigma–Aldrich) supplemented with 5% heat inactivated FBS (Hyclone), 100 U/mL of penicillin, and 0.1 mg/mL streptomycin (Sigma–Aldrich) at 37 °C with 5% CO_2 and 95% air. Cells were kept subconfluent, and media were changed every other day. All cells used were within 3–30 passages. DMSO was used to dissolve the six curcumin analogues, and the final concentration of DMSO in all culture media was 0.1%. PI/RNase

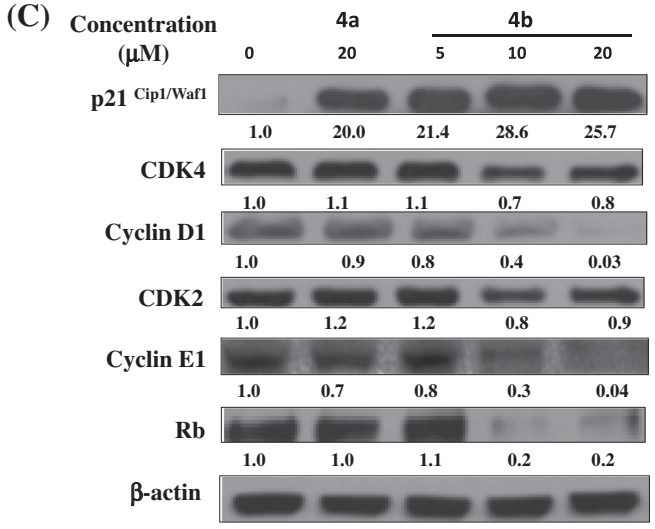


Fig. 5 (*continued*)

staining buffer was purchased from BD Pharmingen. All other chemicals are of analytical grade.

4.2. General methods for chemical synthesis

All starting materials and solvents were obtained from commercial sources and used without further purification. Thin-layer chromatography (TLC) was performed on precoated E. Merck silica-gel 60 F254 plates. Column chromatography was performed on silica gel (200–300 mesh). Melting points were determined on a Mitamura–Riken micro-hot stage and were not corrected. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Jeol JNM-ECP 600 spectrometer with tetramethylsilane (Me_4Si) as the internal standard, and chemical shifts were recorded in δ values. Mass spectra were recorded on a Q-TOF Global mass spectrometer.

4.3. General procedure for the synthesis of compounds 4a–f

4,6-Dimethyl-2-hydroxyl-pyrimidine hydrochloride (1 equiv) and appropriate substituted benzaldehydes (3 equiv) were dissolved in anhydrous ethyl alcohol and toluene in a round-bottomed flask (500 mL). Then concentrated hydrochloric acid (3 equiv) was added and the mixture was refluxed at 100 °C for 36 h. After completion of the reaction, the solvent was evaporated under reduce pressure. The saturated $NaHCO_3$ solution (250 mL) was added into the brown oil residue for desalting. After stirring at room temperature for 5 h, the resulting precipitate was filtered and washed with water and ether to obtain crude product and then purified by chromatography on silica gel (eluent, dichloromethane/methanol) to give **2a**–**b** as brown powder.

4.3.1. (*E*,*E*)-4,6-Bis(3,4-dimethoxystyryl)-2-hydroxyl-pyrimidine (2a)

The titled compound **2a** was obtained from 4,6-dimethyl-2-hydroxyl-pyrimidine hydrochloride (2.18 g, 15 mmol), 3,4-dimethoxybenzaldehyde (7.48 g, 45 mmol), conc HCl (3.75 mL 45 mmol), anhydrous EtOH (150 mL) and toluene (50 mL) according to the procedure described above as brown powder (4.41 g, 70%): R_f = 0.20 ($CHCl_3$/MeOH 9:1); mp: 255–257 °C; ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 11.55 (s, 1H), 7.80–7.77 (d, J = 17.28 Hz, 2H), 7.29 (s, 2H), 7.20 (d, J = 7.74 Hz, 2H), 7.04–7.02 (d, J = 7.92 Hz, 2H), 6.94–6.92 (d, J = 15.42 Hz, 2H), 6.85 (s, 1H), 3.84 (s, 6H), 3.81 ppm (s, 6H); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 151.1, 149.6, 138.8, 128.6, 122.7, 112.3, 110.4, 56.1 ppm; MS (ESI) m/z 421.2 [M+H]$^+$.

4.3.2. (*E*,*E*)-4,6-Bis(3,4,5-trimethoxystyryl)-2-hydroxyl-pyrimidine (2b)

The titled compound **2b** was obtained from 4,6-dimethyl-2-hydroxyl-pyrimidine hydrochloride (2.18 g, 15 mmol), 3,4,5-trimethoxybenzaldehyde (8.83 g, 45 mmol), conc HCl (3.75 mL 45 mmol), anhydrous EtOH (150 mL) and toluene (50 mL) according to the procedure described above as brown powder (4.68 g, 65%): R_f = 0.20 ($CHCl_3$/MeOH 9:1); mp: 222–224 °C, ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 11.71 (s, 1H), 7.79 (d, J = 16.5 Hz, 2H), 6.99 (m, 7H); 3.85 (s, 12H); 3.71 ppm (s, 6H); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 153.7, 139.6, 139.0, 131.4, 107.6, 105.7, 67.2, 60.7, 56.5 ppm; MS (ESI) m/z 481.2 [M+H]$^+$.

General method for the synthesis of compounds (**3a**–**b**). A mixture of compound **2a**–**b** (3.0 mmol) and $POCl_3$ (25 mL) was stirring in a round-bottomed flask (50 mL), then DIPEA (1 mL, 5.8 mmol) was added and the mixture was refluxed at 100 °C for 2 h. After completion of the reaction, the $POCl_3$ was evaporated under reduce pressure and the residue was poured into cold saturated $NaHCO_3$ solution. The precipitate was collected by filtration and washed with water to give crude product. The crude product was purified through column chromatography on silica gel (eluent dichloromethane/methanol) to give compounds **3a**–**b** as yellow powder.

4.3.3. (*E*,*E*)-4,6-Bis(3,4-dimethoxystyryl)-2-chloro-pyrimidine (3a)

The title compound **3a** was obtained from **2a** (1.26 g, 3.0 mmol), $POCl_3$ (25 mL) and DIPEA (1 mL, 5.8 mmol) according to the procedure described above as yellow powder (591 mg, 45%): R_f = 0.90 (CH_2Cl_2/MeOH 20:1); mp: 108–110 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.85 (d, J = 15.9, 2H), 7.16–7.15 (dd, J = 8.22, 1.44, 2H), 7.12–7.11 (d, J = 2.22, 2H), 7.05 (s, 2H), 6.90 (s, 1H), 6.88 (s, 1H), 6.86 (d, J = 15.96, 2H), 3.92 (s, 6H), 3.91 ppm (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 165.8, 161.5, 150.8, 149.3, 138.6, 128.4, 122.5, 122.2, 113.8, 111.2, 109.6, 56.0 ppm; MS (ESI) m/z 439.1 [M+H]$^+$.

4.3.4. (*E*,*E*)-4,6-Bis(3,4,5-trimethoxystyryl)-2-chloro-pyrimidine (3b)

The title compound **3b** was obtained from **2b** (1.44 g, 3.0 mmol), $POCl_3$ (25 mL) and DIPEA (1 mL, 5.8 mmol) according to the procedure described above as yellow powder (672 mg, 45%): R_f = 0.80 (CH_2Cl_2/MeOH 20:1); mp: 104–106 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.85–7.82 (d, J = 15.84, 2H), 7.19 (s, 1H), 6.93–6.91 (d, J = 15.42, 2H), 6.83 (s, 4H), 3.91 (s, 12H), 3.90 ppm (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 165.64, 161.54, 153.57, 139.91, 138.81, 130.89, 123.82, 114.24, 105.11, 56.24 ppm; MS (ESI) m/z 499.1 [M+H]$^+$.

General method for the synthesis of compounds (**4a**–**f**). A mixture of compound **3a**–**b** (1 mmol) and appropriate amine (10 mmol) in anhydrous EtOH (50 mL) was stirred at 90 °C for 12 h and monitored with TLC. When the reaction was finished, the solvent was evaporated under reduce pressure and the residue was purified by column chromatography (silica gel dichloromethane/methanol) to yield the desired compounds.

4.3.5. 4,6-Bis((*E*)-3,4-dimethoxystyryl)-*N*-ethylpyrimidin-2-amine (4a)

The title compound **4a** was obtained from **3a** (0.44 g 1 mmol), ethylamine (3 mL) and anhydrous EtOH (50 mL) according to the procedure described above as yellow powder (232 mg, 52%): R_f = 0.50 (CH_2Cl_2/MeOH 20:1); mp: 74–76 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.71 (d, J = 15.9, 2H), 7.14 (d, J = 6.6, 4H), 6.88 (d,

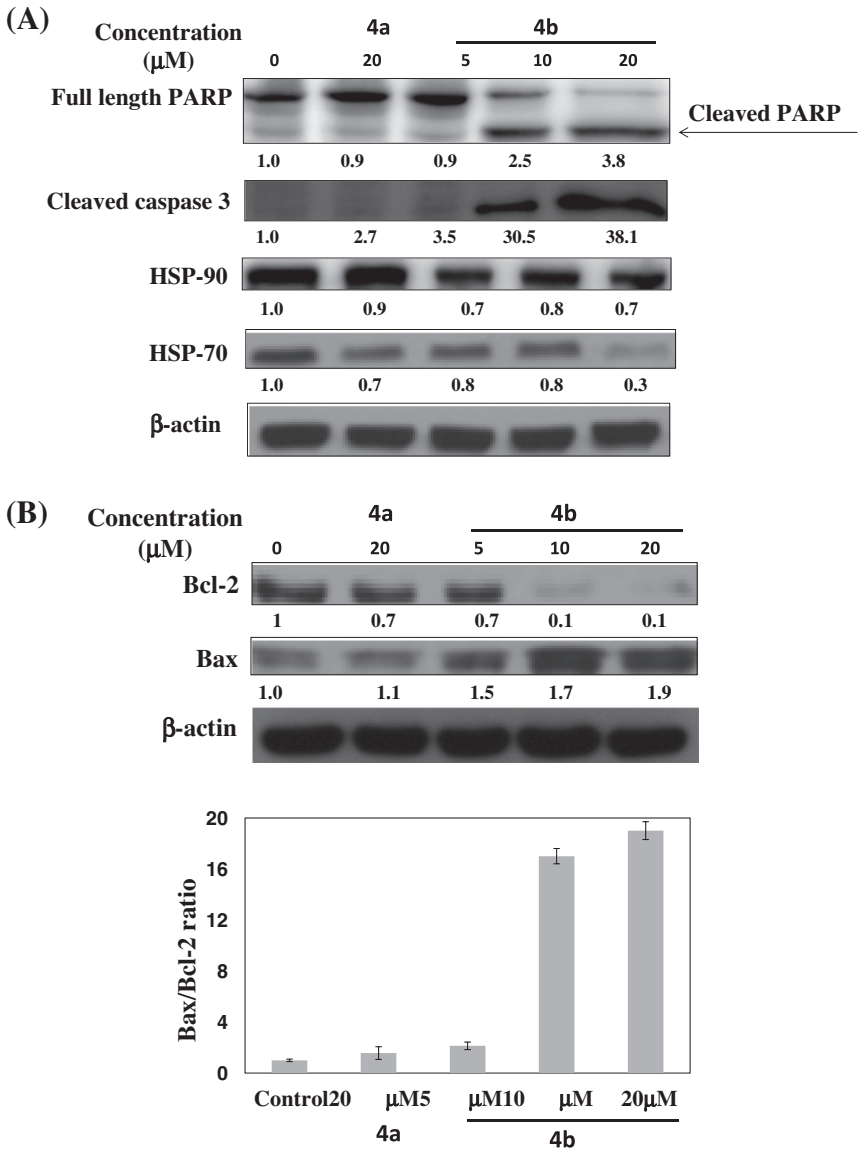


Figure 6. Effects of the curcumin analogues on apoptosis-related proteins in HCT116 human colon cancer cells after treating with the curcumin analogues. HCT116 cells were seeded in 15 cm dishes for 24 h, and then cells were treated with **4a** at 20 μM or **4b** at 5, 10 and 20 μM. After 72 h of incubation, cells were harvested for Western blotting analysis as described in Section 4. The numbers underneath of the blots represent band intensity (normalized to β-actin, means of three independent experiments) measured by Image J software. The standard deviations were all within ±15% of the means (data not shown). β-Actin was served as an equal loading control. The experiments were repeated three times.

J = 8.82, 2H), 6.84 (s, 1H), 6.81 (s, 1H), 6.67 (s, 1H), 5.32 (t, *J* = 6.6, 1H), 3.95 (s, 6H), 3.91 (s, 6H), 3.58 (m, 2H), 1.29 ppm (t, *J* = 8.34, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.70, 162.50, 150.10, 149.22, 135.45, 129.28, 125.03, 121.60, 111.16, 109.39, 106.45, 56.02, 36.45, 15.24 ppm; HRMS *m*/*z* [M+H]$^+$ calculated for $C_{26}H_{30}N_3O_4$: 448.2231, found 448.2225.

4.3.6. 2-(4,6-Bis((*E*)-3,4-dimethoxystyryl)pyrimidin-2-ylamino)ethanol (4b)

The title compound **4b** was obtained from **3a** (0.44 g 1 mmol), 2-aminoethanol (3 mL) and anhydrous EtOH (50 mL) according to the procedure described above as yellow powder (315 mg, 68%): R_f = 0.45 (CH_2Cl_2/MeOH 20:1); mp: 145–147 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.66 (d, *J* = 15.9, 2H), 7.15 (d, *J* = 2.16, 1H), 7.13 (d, *J* = 2.22, 1H), 7.12 (d, *J* = 1.68, 2H), 6.88 (d, *J* = 8.22, 2H), 6.82 (s, 1H), 6.79 (s, 1H), 6.71 (s, 1H), 5.53 (t, *J* = 5.79, 1H), 3.94 (s, 6H), 3.92 (s, 6H), 3.91 (t, *J* = 4.98, 2H), 3.70 ppm (q, *J* = 5.46, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.9, 163.0, 150.3, 149.2, 136.1, 129.0, 124.4, 121.7, 111.2, 109.4, 107.0, 64.7, 56.0, 45.1 ppm; HRMS *m*/*z* [M+H]$^+$ calcd for $C_{26}H_{30}N_3O_5$: 464.2180, found 464.2187.

4.3.7. 4,6-Bis((*E*)-3,4-dimethoxystyryl)-*N*-propylpyrimidin-2-amine (4c)

The title compound **4c** was obtained from **3a** (0.44 g 1 mmol), propylamine (3 mL) and anhydrous EtOH (50 mL) according to the procedure described above as yellow powder (258 mg, 56%): R_f = 0.40 (CH_2Cl_2/MeOH 20:1); mp: 67–69 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.71 (d, *J* = 15.4, 2H), 7.13 (d, *J* = 4.38, 4H), 6.87 (d, *J* = 8.82, 2H), 6.83 (d, *J* = 15.4, 2H), 6.66 (s, 1H), 5.09 (t, *J* = 5.49, 1H), 4.12 (q, *J* = 7.14, 2H), 3.94 (s, 6H), 3.90 (s, 6H), 3.52 (q, *J* = 6.87, 2H), 1.16 (m, 2H), 1.25 (t, *J* = 7.14, 3H), 1.03 ppm (t, *J* = 7.41, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.7, 162.7, 150.0, 149.2, 135.3, 129.3, 125.1, 121.5, 111.2, 109.4, 106.3, 56.0, 43.4, 23.1, 11.7 ppm; HRMS *m*/*z* [M+H]$^+$ calcd for $C_{27}H_{32}N_3O_4$: 462.2387, found 461.2382.

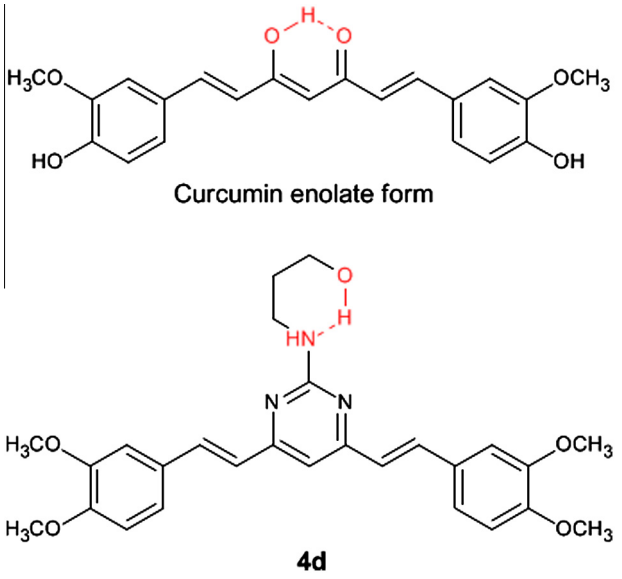


Figure 7. Enolate form of curcumin might be critical for EGFR regulating activities.

4.3.8. 3-(4,6-Bis((*E*)-3,4-dimethoxystyryl)pyrimidin-2-ylamino)propan-1-ol (4d)

The title compound **4d** was obtained from **3a** (0.44 g 1 mmol), 3-amino-1-propanol (3 mL) and anhydrous EtOH (50 mL) according to the procedure described above as yellow powder (253 mg, 53%): R_f = 0.34 (CH_2Cl_2/MeOH 20:1); mp: 142–144 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.64 (d, J = 15.9, 2H), 7.14 (d, J = 1.62, 1H), 7.12 (s, 1H), 7.11 (s, 2H), 6.87 (d, J = 8.28, 2H), 6.81 (d, J = 15.9, 2H), 6.7 (s, 1H), 5.56 (t, J = 6.6, 1H), 3.93 (s, 6H), 3.90 (s, 6H), 3.72(q, J = 6.33, 2H), 3.68 (t, J = 5.52, 2H), 1.79 ppm (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.9, 163.0, 150.3, 149.2, 136.1, 128.9, 124.3, 121.7, 111.1, 109.4, 106.2, 58.4, 55.9, 37.2, 33.3 ppm; HRMS m/z [M+H]$^+$ calcd for $C_{27}H_{32}N_3O_5$: 478.2336, found 478.2330.

4.3.9. 4,6-Bis((*E*)-3,4,5-trimethoxystyryl)-*N*-propylpyrimidin-2-amine (4e)

The title compound **4e** was obtained from **3b** (0.50 g 1 mmol), propylamine (3 mL) and anhydrous EtOH (50 mL) according to the procedure described above as yellow powder (339 mg, 65%): R_f = 0.35 (CH_2Cl_2/MeOH 20:1); Mp:102–104 °C; ^{1}H NMR ($CDCl_3$, 600 M) δ 7.69 (2H, d, J = 15.9), 6.87 (2H, d, J = 15.9), 6.81 (4H, s), 6.70 (1H, s), 5.11–5.10 (1H, t, J = 5.22), 3.91–3.88 (18H, s), 3.51–3.50 (2H, q, J = 6.6), 1.62–1.67 (2H, m), 1.05–1.01 (3H, t, J = 7.41) ppm; ^{13}C NMR ($CDCl_3$, 150 M) δ 163.5, 162.7, 153.4, 139.0, 135.5, 131.8, 126.4, 106.5, 104.6, 61.0, 56.2, 43.4, 23.1, 11.7 ppm; HRMS (ESI) m/z calcd for $C_{29}H_{36}N_3O_6^+$ 522.2599, found 522.2584.

4.3.10. 3-(4,6-Bis((*E*)-3,4,5-trimethoxystyryl)pyrimidin-2-ylamino)propan-1-ol (4f)

The title compound **4f** was obtained from **3b** (0.50 g 1 mmol), 3-amino-1-propanol (3 mL) and anhydrous EtOH (50 mL) according to the procedure described above as yellow powder (376 mg, 70%): R_f = 0.30 (CH_2Cl_2/MeOH 20:1); mp: 102–104 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.64 (d, J = 15.9, 2H), 6.88 (d, J = 15.36, 2H), 6.81 (s, 4H), 6.74 (s, 1H), 5.30–5.28 (t, J = 6.87, 1H), 3.91 (s, 12H), 3.89 (s, 6H), 3.76–3.73 (q, J = 6.22, 2H), 3.69–3.68 (t, J = 5.22, 2H), 1.83–1.79 ppm (m, 2H); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 163.18, 153.51, 139.35, 136.22, 131.59, 125.95, 106.82, 104.78, 61.08, 58.42, 56.25, 37.24, 33.42 ppm; HRMS m/z [M+H]$^+$ calcd for $C_{29}H_{36}N_3O_7$: 538.2548, found 538.2537.

4.4. Cellular viability assay

For cellular viability assay, a 96-wells plate (Nest) was seeded with 2000 cells/well in complete medium. After 24 h, the medium was removed and 200 μL complete medium containing different amount of drugs was added to each well. The cells were incubated for 48 h, followed by adding 20 μL of resazurin (2 mg/mL dissolved in water, catalog no. R7017-5G, Sigma) to the media for 16 h. The fluorescent signal was monitored using 544 nm excitation wavelength and 595 nm emission wavelength by Spectramax M5 plate reader (Molecular Devices). The relative fluorescence unit (RFU) generated from the assay was proportional to the number of living cells in each well.[28] The IC_{50} value of each drug was calculated by the Logit approach.

4.5. Cell cycle analyses

HCT116 and HT29 cells were seeded in 6-well plates (4×10^4 cells/ml, 2 ml/well). After 24 h incubation for attachment, cells were treated with or without different amount of **4a** or **4b** in 2 ml of complete media. After another 24 h, media was collected and combined with adherent cells that were detached by brief trypsinization (0.25% trypsin-EDTA; Sigma–Aldrich). Cell pellets were washed with 1 ml of ice-cold PBS two times and then resuspended in 1 ml of 70% ethanol in 4 °C overnight. After centrifugation (1600*g*, 1 min), the supernatant was removed and cells were incubated with 0.5 ml of PI/RNase staining buffer for 15 min at room temperature. Single-cell suspension was generated by gentle pipetting. Cell cycle was analyzed using a FC500 MCL/MPL cell analyzer and data were processed using MultiCycle software.[29]

4.6. Whole cell lysate preparation

Cells were seeded in cell culture dishes at a density of 8×10^4 - cells/ml and 20 ml per dish in McCoy's 5A medium containing 5% FBS for 24 h. Different amount of **4a** or **4b** were then added directly to the medium of the cultured cells and incubated for another 72 h. Cells were rinsed with PBS and total cell lysates were prepared by adding 200 μL of whole cell lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin (pH 7.5) to each dish and collected with cell scrapers into microcentrifuge tubes. Cell suspensions were then subjected to sonication (5s, three times). After further incubation for 30 min, supernatants were collected by centrifugation at 10,000*g* for 10 min.[30] Protein concentrations were determined by BCA protein assay kit (Beyotime Institute of Biotechnology, China), following manufacturer's instruction.

4.7. Preparation of cell membrane fraction

Trypsinized cells were washed with ice-cold PBS and then incubated in the presence of buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1 mM DTT) containing protease inhibitor cocktail (1 mM Na_3VO_4, 1 mM leupeptin, 1 mM PMSF). The cell suspension was sonicated 3s on ice four times followed by centrifugation at 100,000*g* for 1 h. The pellets were solubilized with lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate) supplemented with protease inhibitor cocktail (1 mM Na_3VO_4, 1 mM leupeptin, 1 mM PMSF) on ice for 30 min after thorough mixing. After centrifugation at 10,000*g* for 30 min, the cell membrane fraction present in supernatant was collected.[29]

4.8. Western blotting assay

For immunoblot analyses, equal amounts of proteins (20–300 μg, depending on the proteins of interest) were subjected to 12% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer (Tris–buffered saline-0.05% Tween-20 (TBST) containing 5% nonfat milk) at room temperature for 1 h, then incubated with appropriate antibodies according to the protocols of the manufacturers and washed with TBST. After incubation with appropriate secondary antibodies, the membranes were washed three times with Tris–buffered saline containing Tween-20 and then visualized using enhanced chemiluminescence. Antibodies for PARP, BAX, Bcl-2, HSP-70, HSP-90, $p21^{WAF-1/CIP-1}$, P-EGFR (Tyr1068), Rb, cyclinD1, cleaved caspase-3, caspase-8, CDK-2, CDK-4 and β-actin were obtained from Cell Signaling Technology, Inc. Antibodies for EGFR, cyclin E1, β-tubulin were purchased from Immunoway, Inc.

Acknowledgments

This work was supported by Natural Science Foundation of China (Grant Nos. 21171154 and 91129706), Special Fund for Marine Scientific Research in the Public Interest (01005024), and Program for Science and Technology Development of Shandong Province (Grant No. 2012GSF11912).

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